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## On the Localisation of d-Tubocurarine in Rat Liver Lysosomes *in vivo* by Electron Microscopy and Subcellular Fractionation

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**Summary.** After i.v. injection in the rat, d-tubocurarine is taken up and concentrated by the liver. A method is developed for the visualisation of d-tubocurarine inside the liver cell by electron microscopy. Glutaraldehyde fixed liver blocks were immersed in an ammonium molybdate solution; d-tubocurarine was precipitated at sites of high concentration by molybdate, to form an insoluble d-tubocurarine-molybdate complex. This precipitate was found predominantly at the surface of lysosome-like particles, but also inside these organelles. In subcellular fractionation experiments, d-tubocurarine was found with a high relative specific "activity" in the lysosomal fraction, lending support to a lysosomal localisation of d-tubocurarine.

**Key words:** d-Tubocurarine — Subcellular Distribution — Lysosomes — Electron Microscopy.

After i.v. administration many drugs are rapidly taken up in the liver of the rat and, subsequently, may be subject to biotransformation, stored in the liver, or excreted in bile. In this process, the concentration of the drug in the liver (moles/gram of liver) may become higher than the concentration in blood, as has been found for example with d-tubocurarine (dTc) (Meijer *et al.*, 1972). This may be due to accumulation of this quaternary ammonium compound in, or at some subcellular particle. One of the main problems in the localisation of drugs inside the intact liver cell is that in subcellular fractionation experiments many drugs, due to their high lipid solubility, are extensively bound to particles; this binding need not be related to the subcellular distribution of the drug *in vivo*, but may be due to the redistribution after homogenisation (unpublished results). Therefore we developed an electron microscopic method for visualisation of dTc inside the intact liver cell. Together with the outcome of the subcellular fractionation experiments, the present results suggest that the drug is concentrated in, or at the surface of liver lysosomes.

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## Materials and Methods

*d-Tubocurarine.* Commercial  $^3\text{H}$ -d-tubocurarine was further purified according to the method of Cohen *et al.* (1967) as modified by Meijer *et al.* (1972).

*Subcellular Fractionation.* Male rats (Wistar, about 300 g) had free access to food and water, since in previous experiments on the biliary excretion of dTc (Meijer *et al.*, 1972) also non-fasted had been used. The rats were anaesthetised with pentobarbital (Nembutal®; 60 mg/kg) i.p. and the kidneys were ligated. A dose of 2.6  $\mu\text{moles}$  dTc/kg (dissolved in 0.9% w/v NaCl in water), or saline alone (in the blanks) was injected into the vena jugularis externa. After 15 min the liver was excised and put into icecold 0.15 M sucrose, pH 7.0. A 20% (w/v) homogenate was made using a Potter Elvehjem homogeniser. The subcellular fractions were prepared according to the method of De Duve *et al.* (1955) as described by Bouma and Gruber (1966). In control experiments, animals received no dTc *in vivo*, but the amount of dTc, to be expected in the liver 15 min after its i.v. injection, was added to the liver in the icecold homogenisation medium; the liver was subsequently homogenised in the presence of dTc.

*Determinations.* Acid phosphatase was determined according to Gianetto and de Duve (1955). Protein was determined by the Lowry method, with bovine serum albumin as standard (Lowry *et al.*, 1951).  $^3\text{H}$  dTc was determined as described before (Meijer *et al.*, 1972).

*Electron Microscopy of dTc in Rat Liver.* Non-fasted rats were treated as described above. 15 min after injection of the dTc solution or the saline, the liver was perfused through the portal vein with 1.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 min at room temperature. The tissue was excised and small blocks were immersed for 1 hr in a 1% (w/v) solution of ammonium heptamolybdate in twice distilled water. The molybdate-dTc interaction resulted in a finely dispersed precipitate. Its visualisation was improved by keeping tissue contrast low. The tissue was therefore postfixed for only 1 hr in cacodylate buffered osmium tetroxide (1% w/v) at 4° C. Poststaining with metal salts was omitted for the same reason. The blocks were dehydrated in graded series of ethanol and in propylene oxide, and embedded in Epon 812. Ultra-thin sections were cut with a LKB Ultratome and examined unstained on unsupported grids in a Philips EM 300 electron microscope.

## Results

### *Precipitation of d-Tubocurarine by Molybdate*

In a pilot experiment we found that, out of a number of heavy metal salts, ammonium heptamolybdate most completely precipitated dTc from a solution in physiological saline *in vitro*. It seemed to form a complex, resulting in an insoluble white precipitate (Table 1). The results from Table 1 suggest that more than one molecule of dTc complexes with each molecule of molybdate. Similar precipitates were obtained with ammonium metavanadate, dodeca tungsto-phosphoric acid ( $\text{H}_3[\text{P}(\text{W}_3\text{O}_{10})_4]$ ) and dodeca tungsto-silicic acid ( $\text{H}_4[\text{Si}(\text{W}_3\text{O}_{10})_4]$ ). This complexing reaction of dTc with molybdate has also been described by Clarke (1969).

### *Electron Microscopy of dTc in Rat Liver*

To visualise dTc in the liver cell by electron microscopy we tried to use the precipitation reaction with molybdate in order to obtain an

Table 1. Precipitation of dTc by ammonium molybdate

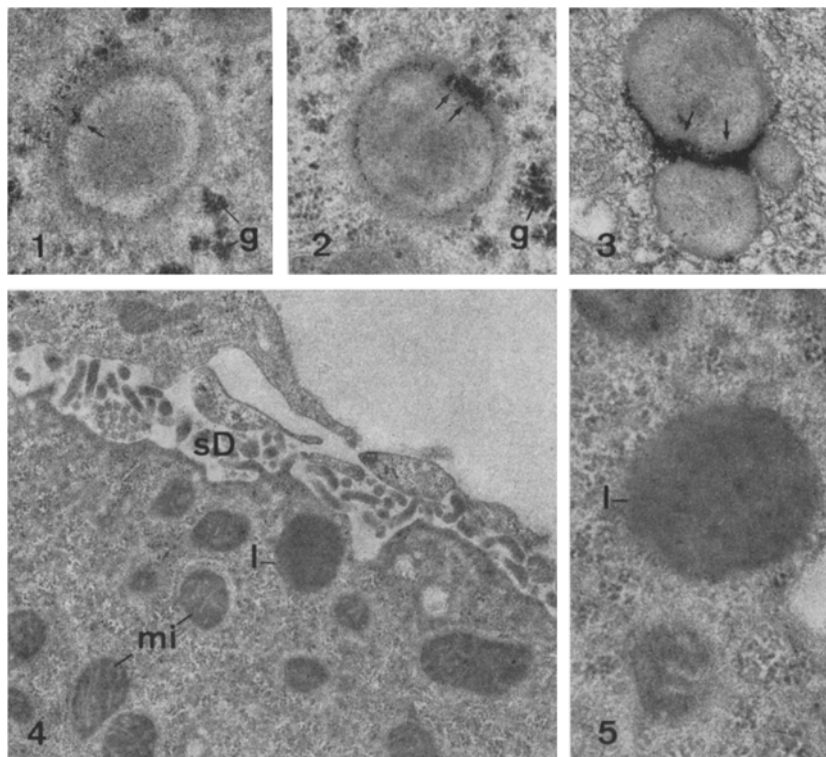
Ammonium heptamolybdate (mM)	percent $^3\text{H}$ remaining in supernatant
0.1	48
0.5	30
1.0	15
2.5	1
5.0	1
10.0	1

To a 2.5 mM solution of  $^3\text{H}$  dTc in physiological saline (pH 7.0) various amounts of ammonium heptamolybdate were added, resulting in the final ammonium heptamolybdate concentrations given in the left column. The precipitate was spun down and after centrifugation the percentage of  $^3\text{H}$  remaining in the supernatant was determined. 100% corresponds to about 300 000 dpm/ml.

electron dense deposit at sites of a high dTc concentration. Liver tissue blocks of rats that had received dTc intravenously were treated with an ammonium molybdate solution (after fixation with glutaraldehyde). The electron micrographs (Figs.1—3) show that in the dTc treated rats a precipitate can be found in association with particles that may be lysosomes. This precipitate is only found in the liver of dTc treated rats; further, it is found only after molybdate treatment (Figs.4 and 5). Therefore, we tentatively assume that the precipitate represents a dTc-molybdate sediment at sites of high concentration of dTc in the liver cell. In the figures several typical examples of the dTc-molybdate precipitate are given. The results suggest that the precipitate is preferentially located at the membrane-surface of the particles, although it is found also inside the particles.

#### *Subcellular Distribution of $^3\text{H}$ dTc*

In subcellular fractionation experiments we observed that  $^3\text{H}$  dTc, after i.v. administration, showed a distribution similar to that of acid phosphatase, a marker enzyme of the lysosomal fraction. Comparison of Fig.6A and 6B clearly shows that the accumulation of  $^3\text{H}$  dTc in the lysosomal fraction occurs only, if  $^3\text{H}$  dTc is administered to the liver by the *in vivo* route (6A); when it was added to the liver homogenate *in vitro*, the dTc concentration in the lysosomal fraction was not enhanced but the compound was distributed nearly homogenously over all fractions (6B). About 16% of the total amount present in the liver after 15 min (0.09  $\mu\text{moles}$ ) was found in the lysosomal fraction. Thus after i.v. injection,  $^3\text{H}$  dTc is (partly) associated with particulate matter sedi-



Figs. 1—3. Liver cells of dTc treated rats. After fixation the liver tissue blocks were treated with an ammonium heptamolybdate solution. Small electron dense particles are found in lysosome-like bodies. They are especially concentrated at the periphery of these organelles (arrows). *g* glycogen.  $\times 42000$

Figs. 4 and 5. Untreated rat liver cell. After fixation the liver tissue blocks were treated with an ammonium molybdate solution. *sD* space of Disse; *l* lysosome; *mi* mitochondria.  $\times 19000$ . Fig. 5 is a detail of Fig. 4. No dense deposits are found in the lysosome (*l*).  $\times 47000$

menting in the lysosomal fraction. Of course, it cannot be concluded whether it is taken up by a particle, presumably a lysosome, or bound to its surface.

### Discussion

In the present work a method is presented that may become a tool in the study of the subcellular localisation of drugs. dTc is precipitated in the form of an electron dense dTc-molybdate complex. Although at present no data are available on the nature of this complex, it seems likely that it is an ion-pair formation between cationic dTc and anionic

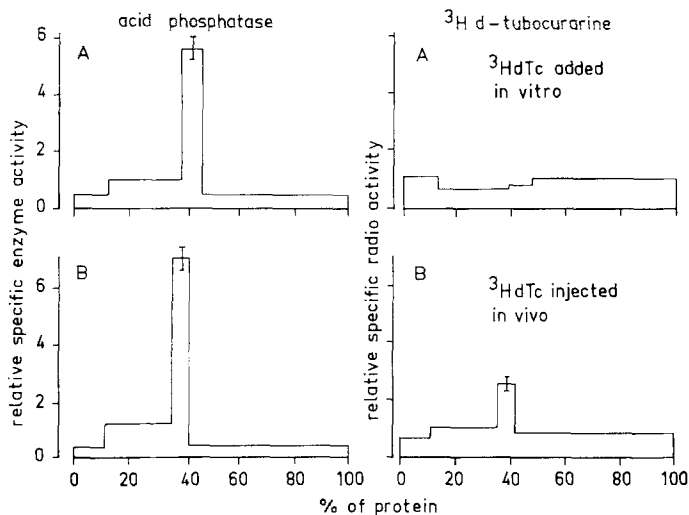


Fig. 6. Distribution pattern of acid phosphatase and  $^3\text{H}$  dTc after fractionation of rat liver by differential centrifugation. The rats received an i.v. injection of  $2.6 \mu\text{moles } ^3\text{H dTc/kg}$  (" $^3\text{H dTc in vivo}$ " group) and the liver was excised after 15 min (B); in control (" $in vitro$ ") experiments (A)  $0.09 \mu\text{moles } ^3\text{H dTc/kg}$  were added to the  $0.25 \text{ M}$  sucrose into which the liver of an untreated rat was put after excision. Fractions are plotted in the order of the average coefficient of sedimentation of their subcellular components, i.e. from left to right: nuclear, mitochondrial, lysosomal and postlysosomal supernatant. Each fraction is represented separately in the ordinate scale by the relative specific activity of the constituent (percentage of the total amount/percentage of total protein). On the abscissa each fraction is represented by its percentage of total protein. Mean values of 3 experiments are given; the SEM is indicated only for the lysosomal fraction

molybdate. The same reaction is used as identification reaction for dTc (Clarke, 1969).

Results from subcellular fractionation are combined with electron microscopic evidence to suggest that dTc is, in part, bound to or taken up in lysosomes. It is unlikely, although it cannot be excluded completely, that the pertinent particles might be fat droplets or microbodies. In order to visualise the dTc-molybdate precipitate, membrane contrast had to be kept low and consequently many membranes were poorly contrasted. Although this complicates the analysis of the electron micrographs, the results from the subcellular fractionation lend support to a lysosomal localisation of dTc after its *in vivo* injection.

When dTc is added *in vitro* to the liver, a high affinity is found for binding to all particles (Fig. 6). This phenomenon masks the interactions with specific particles which take place when the drug is administered *in vivo*. After homogenisation of the liver, the drug in the intact cell will be

redistributed to an unknown extent, presumably in a manner similar to the distribution of dTc, when added *in vitro*. Nevertheless, a distinct increase in relative specific radioactivity of  $^3\text{H}$  dTc in the lysosomal fraction is observed in the *in vivo* treated liver. The electron microscopical data show a localisation of dTc in lysosome-like particles. It is not yet clear why the dTc-molybdate complex is predominantly located at the outside of the particles, as is indicated in Figs. 1—3. One explanation for this type of location may be that dTc is primarily deposited at the outside when applied *in vivo*; alternatively, dTc may be distributed uniformly in the lysosome-like bodies and diffuse out of the particle during the incubation with molybdate which causes the complex to precipitate at the outside of the particles. This problem as well as the nature of the particles concerned require further investigation. The present results show that information can be obtained by an electron microscopical method on the subcellular distribution of dTc (and possibly other related cationic compounds) at a level which is inaccessible to conventional biochemical fractionation methods. The significance of the observed binding of dTc to lysosome-like bodies for handling of dTc by the liver remains to be investigated.

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